

SYSTEM AND METHOD FOR ON-SITE DETECTION OF PATHOGENS

5 TECHNICAL FIELD

The present disclosure relates broadly to a system and method for on-site detection of pathogens.

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BACKGROUND

Water is essential to life on Earth and ensuring the safety of water sources is important for public health. Unsafe drinking water, poor sanitation and lack of hygiene are significant causes of disease in both developing and developed nations. Rapid and early monitoring and detection of pathogens e.g. bacteria in water sources are thus vital to mitigate the risks involved in unsafe water sources.

20 Currently, detection of water-borne bacteria relies predominantly on standard culture methods. Typically, culture methods exclusively detect for culturable live bacteria rather than dead bacteria. Dead bacteria generally do not pose any significant risk to the public health and hence are not of particular concern for the regulatory bodies. As a result, the culture method remains as the gold standard for detection of bacteria. However, known culture methods have slow result turnover and are unable to detect for certain types of live bacteria that are not culturable or are difficult to culture in vitro. Typical time-to-result of culture methods can take as long as from a few days to about 2 weeks, depending on the bacteria of interest. This creates a significant gap in the current water safety monitoring system where timely and efficient reporting can minimise public exposure to contaminated water

sources, such as drinking water, recreational water (e.g. swimming pool, water playground, spa, etc), natural water bodies, air-con cooling towers in commercial buildings, etc.

5 Existing commercially available rapid point-of-care water testing kits generally have low levels of sensitivity and laboratory-based molecular detection method such as PCR (Polymerase Chain Reaction) are generally not widely used due to the associated cost and relatively high level of technical expertise required.

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Furthermore, existing methods or kits for detection usually detect both live and dead bacteria and as a result may give rise to false positive results. Although there are specific test reagents that can be used to minimise false positive result, especially in the case of PCR, but because of the complexity and costly operation of PCR, its application as a detection system in water safety monitoring remains limited.

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In light of the aforementioned deficiencies, in particular the long time-to-results turnover of culture method, complex and costly operation of PCR and low-level sensitivity of rapid kits, existing kits and methods are not suitable to replace or even to complement the culture method as an early-warning system.

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Thus, there is a need for a system and method for on-site detection of pathogens that seek to address or at least ameliorate one of the above problems.

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SUMMARY

According to one aspect, there is provided an on-site detection system for detecting pathogens in a liquid sample, the system comprising: a filtration
5 module comprising a mixture of dead-end filtration elements and cross-flow filtration elements configured to obtain a sample population of pathogens from the liquid sample, an amplification module configured to amplify one or more target biological elements present in one or more target species of pathogens in the sample population of pathogens; and a detection module
10 configured to detect presence of amplified products of the one or more target biological elements present in the one or more target species of pathogens, wherein a positive reading on the detection module indicates the presence of the one or more target species of pathogens.

15 In one embodiment, the system further comprises a treatment module comprising a composition for preferentially interacting with one or more biological elements present in non-viable pathogens in the sample population of pathogens to render said one or more biological elements incapable of amplification.

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In another embodiment, the pathogens are selected from the group consisting of bacteria, viruses, parasites and fungi.

25 In another embodiment, the one or more biological elements comprise DNA.

In another embodiment, the composition for preferentially interacting with one or more biological elements comprises propidium monoazide or analogs thereof.

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In another embodiment, the amplification module comprises recombinase polymerase amplification of the one or more target DNAs from

the one or more target species of pathogens in the sample population of pathogens.

5 In another embodiment, the amplification module comprises a probe and at least one primer, wherein the probe and the at least one primer comprise a nucleotide sequence having at least 80% sequence identity to a sequence selected from the group consisting of SEQ ID NO. 1 to SEQ ID NO. 5, for detecting *Pseudomonas aeruginosa*.

10 In another embodiment, the amplification module comprises a probe and at least one primer, wherein the probe and the at least one primer comprise a nucleotide sequence having at least 80% sequence identity to a sequence selected from the group consisting of SEQ ID NO. 6 to SEQ ID NO. 10, for detecting *Legionella pneumophila*.

15 In another embodiment, the amplification module comprises a probe and at least one primer, wherein the probe and the at least one primer comprise a nucleotide sequence having at least 80% sequence identity to a sequence selected from the group consisting of SEQ ID NO. 11 to SEQ ID NO. 15, for detecting *Enterococcus* species.

20 In another embodiment, the amplification module further comprises a freeze-dried enzyme mixture, a rehydration buffer, a solution of magnesium acetate, a labelled probe, a positive control template DNA and water.

25 In another embodiment, the amplification module further comprises one or more portable heating devices for providing one or more operating temperatures to perform amplification.

30 In another embodiment, the detection module comprises a lateral flow strip configured to detect labelled amplified products.

In another embodiment, the detection module comprises a fluorescence detector configured to detect fluorescence-labelled amplified products.

5 In another embodiment, on-site detection of pathogens is performed in less than 90 minutes.

In another embodiment, the system has a sensitivity of at least 80%.

10 In another embodiment, the system has a specificity of at least 75%.

In another embodiment, the system is capable of detecting pathogens in the liquid sample at a concentration range from 0.8 to 1.5 CFU per 100 ml.

15 According to another aspect, there is provided an on-site method for detecting pathogens in a liquid sample, the method comprising, filtering the liquid sample with a filtration module comprising a mixture of dead-end filtration elements and cross-flow filtration elements to obtain a sample population of pathogens; amplifying one or more target biological elements
20 present in one or more target species of pathogens in the sample population of pathogens; and detecting the presence of amplified products of the one or more target biological elements present in the one or more target species of pathogens.

25 In one embodiment, the method further comprises treating the sample population of pathogens with a composition for preferentially interacting with one or more biological elements present in non-viable pathogens in the sample population of pathogens to render said one or more biological elements incapable of amplification.

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In another embodiment, the pathogens are selected from the group consisting of bacteria, virus, parasites and fungi.

In another embodiment, the one or more biological elements comprise DNA.

5 In another embodiment, the step of treating the sample population of pathogens with a composition comprises exposing the sample population of pathogens to propidium monoazide or analogs thereof.

10 In another embodiment, the step of amplifying comprises performing recombinase polymerase amplification of the one or more target biological elements from the one or more target species of pathogens in the sample population of pathogens.

15 In another embodiment, the step of amplifying comprises using a probe and at least one primer, wherein the probe and the at least one primer comprise a nucleotide sequence having at least 80% sequence identity to a sequence selected from the group consisting of SEQ ID NO. 1 to SEQ ID NO. 5, for detecting *Pseudomonas aeruginosa*.

20 In another embodiment, the step of amplifying comprises using a probe and at least one primer, wherein the probe and the at least one primer comprise a nucleotide sequence having at least 80% sequence identity to a sequence selected from the group consisting of SEQ ID NO. 6 to SEQ ID NO. 10, for detecting *Legionella pneumophila*.

25 In another embodiment, the step of amplifying comprises using a probe and at least one primer, wherein the probe and the at least one primer comprise a nucleotide sequence having at least 80% sequence identity to a sequence selected from the group consisting of SEQ ID NO. 11 to SEQ ID NO. 15, for detecting *Enterococcus* species.

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In another embodiment, the step of amplifying comprises using a freeze-dried enzyme mixture, a rehydration buffer, a solution of magnesium acetate, a labelled probe, a positive control template DNA and water.

5 In another embodiment, the step of amplifying comprises providing one or more operating temperatures with one or more portable, battery-operated heating devices.

10 In another embodiment, the step of detecting comprises using a lateral flow strip configured to detect labelled amplified products.

In another embodiment, the step of detecting comprises using a fluorescence detector configured to detect fluorescence-labelled amplified products.

15 In another embodiment, the method comprises on-site detection of pathogens in less than 90 minutes.

In another embodiment, the method has a sensitivity of at least 80%.

20 In another embodiment, the method has a specificity of at least 75%.

In another embodiment, the method is capable of detecting pathogens in the liquid sample at a concentration range from 0.8 to 1.5 CFU per 100 ml.

25 In another embodiment, the said at least one pair of primers in the system or method as disclosed herein comprises a pair of primers.

30 DEFINITIONS

The term “pathogen” as used herein refers to an agent that causes disease or illness to its host. A pathogen includes but is not limited to bacteria, virus, protozoa and fungi.

5 The term “on-site” as used herein refers to performance of an activity at a site of particular concern. In various embodiments, such sites of particular concern are sites where the sample to be tested are obtained from their resting state. For example, when the sample to be tested are water samples, these sites include but are not limited to reservoirs, ponds, sea, rivers etc from which the
10 samples are obtained from.

 The term “viable” as used herein refers to the ability of cells to replicate. The term as used herein also includes cells which are alive and have substantially intact, non-compromised cell membranes.

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 The term “non-viable” as used herein refers to cells that are not capable of replicating under any known conditions. The term as used herein also includes cells which are dead and have compromised cell membranes.

20 The term “amplification” as used herein refers to a technique of increasing the number of copies of a nucleic acid molecule. For example, amplification of a nucleic acid molecule (e.g. DNA or RNA molecule) includes the use of a technique that increases the number of copies of a nucleic acid molecule in a sample. The resulting amplification products may be termed as “amplicons.”

25 Examples of amplification techniques include but are not limited to polymerase chain reaction (PCR); real time PCR; reverse transcriptase PCR (RT-PCR); real time reverse transcriptase PCR (real time RT-PCR) and recombinase polymerase amplification (RPA). Product of amplification may be characterized/detected by techniques such as lateral flow systems e.g. lateral
30 flow immunoassay, electrophoresis, restriction endonuclease cleavage patterns, oligonucleotide hybridization or ligation, and/or nucleic acid sequencing.

The term "nucleic acid" as used herein refers to a deoxyribonucleotide or ribonucleotide polymer including without limitation, cDNA, mRNA, genomic DNA, viral genome RNA, and synthetic DNA or RNA. The nucleic acid can be double stranded (ds) or single stranded (ss). Where single stranded, the nucleic acid can be the sense strand or the antisense strand. Nucleic acids may include natural nucleotides (such as A, T/U, C, and G), and may also include analogs of natural nucleotides, such as labeled nucleotides.

The term "primer" as used herein refers to an oligonucleotide capable of selectively binding to a specified target nucleic acid or template by hybridizing with the template. A primer is typically between about 10 to 100 nucleotides in length. A primer may provide a point of initiation for template-directed synthesis of a polynucleotide complementary to the template, which can take place under suitable conditions such as in the presence of appropriate enzyme(s), cofactors, substrates such as nucleotides and oligonucleotides and the like.

The term "probe" as used herein refers to an oligonucleotide capable of selectively binding to a specified target nucleic acid or template by hybridizing with the template and has a detectable portion thereon. A probe may be used as a proxy to monitor the amplification of a target nucleic acid during amplification reaction.

The term "label" as used herein refers to any moiety that comprises one or more appropriate substances, which directly or indirectly generate a detectable compound or signal, for example, in a chemical, physical or enzymatic reaction.

The term "abasic residue" as defined herein in an oligonucleotide refers to a molecular fragment within an oligonucleotide chain where the molecular fragment approximates the length of a ribofuranose or a deoxyribofuranose sugar in such a way that bases adjacent to the molecular fragment are separated from one another by the same, or effectively the same, distance as if a ribofuranose

or a deoxyribofuranose sugar of any of A, G, C, T, or U were present in place of the abasic residue.

5 The term "micro" as used herein is to be interpreted broadly to include dimensions from about 1 micron to about 1000 microns. Exemplary sub-ranges that fall within the term include but are not limited to the ranges of from about 10 micron to about 900 microns, from about 20 micron to about 800 microns, from about 30 micron to about 700 microns, from about 40 micron to about 600 microns, from about 50 micron to about 500 microns, from about 60 micron to about 400 microns, from about 70 micron to about 300 microns, from about 80 micron to about 200 microns, or from about 90 micron to about 100 microns.

15 The term "nano" as used herein is to be interpreted broadly to include dimensions less than about 1000 nm. Exemplary sub-ranges that fall within the term include but are not limited to the ranges of less than about 900 nm, less than about 800 nm, less than about 700 nm, less than about 600 nm, less than about 500 nm, less than about 400 nm, less than about 300 nm, less than about 200 nm, less than about 100 nm, less than about 50 nm, less than about 20 nm, or less than about 10 nm.

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The term "particle" as used herein broadly refers to a discrete entity or a discrete body. The particle described herein can include an organic, an inorganic or a biological particle. The particle used described herein may also be a macro-particle that is formed by an aggregate of a plurality of sub-particles or a fragment of a small object. The particle of the present disclosure may be spherical, substantially spherical, or non-spherical, such as irregularly shaped particles or ellipsoidally shaped particles. The term "size" when used to refer to the particle broadly refers to the largest dimension of the particle. For example, when the particle is substantially spherical, the term "size" can refer to the diameter of the particle; or when the particle is substantially non-spherical, the term "size" can refer to the largest length of the particle.

The terms "coupled" or "connected" as used in this description are intended to cover both directly connected or connected through one or more intermediate means, unless otherwise stated.

5 The term "associated with", used herein when referring to two elements refers to a broad relationship between the two elements. The relationship includes, but is not limited to a physical, a chemical or a biological relationship. For example, when element A is associated with element B, elements A and B may be directly or indirectly attached to each other or element A may contain
10 element B or vice versa.

 The term "adjacent" used herein when referring to two elements refers to one element being in close proximity to another element and may be but is not limited to the elements contacting each other or may further include the elements
15 being separated by one or more further elements disposed therebetween.

 The term "and/or", e.g., "X and/or Y" is understood to mean either "X and Y" or "X or Y" and should be taken to provide explicit support for both meanings or for either meaning.

20 Further, in the description herein, the word "substantially" whenever used is understood to include, but not restricted to, "entirely" or "completely" and the like. In addition, terms such as "comprising", "comprise", and the like whenever used, are intended to be non-restricting descriptive language in that they broadly
25 include elements/components recited after such terms, in addition to other components not explicitly recited. Therefore, in embodiments disclosed herein using terms such as "comprising", "comprise", and the like, it will be appreciated that these embodiments provide teaching for corresponding embodiments using terms such as "consisting", "consist", and the like. Further, terms such as "about",
30 "approximately" and the like whenever used, typically means a reasonable variation, for example a variation of +/- 5% of the disclosed value, or a variance

of 4% of the disclosed value, or a variance of 3% of the disclosed value, a variance of 2% of the disclosed value or a variance of 1% of the disclosed value.

Furthermore, in the description herein, certain values may be disclosed in a range. The values showing the end points of a range are intended to illustrate a preferred range. Whenever a range has been described, it is intended that the range covers and teaches all possible sub-ranges as well as individual numerical values within that range. That is, the end points of a range should not be interpreted as inflexible limitations. For example, a description of a range of 1% to 5% is intended to have specifically disclosed sub-ranges 1% to 2%, 1% to 3%, 1% to 4%, 2% to 3% etc., as well as individually, values within that range such as 1%, 2%, 3%, 4% and 5%. The intention of the above specific disclosure is applicable to any depth/breadth of a range.

Additionally, when describing some embodiments, the disclosure may have disclosed a method and/or process as a particular sequence of steps. However, unless otherwise required, it will be appreciated that the method or process should not be limited to the particular sequence of steps disclosed. Other sequences of steps may be possible. The particular order of the steps disclosed herein should not be construed as undue limitations. Unless otherwise required, a method and/or process disclosed herein should not be limited to the steps being carried out in the order written. The sequence of steps may be varied and still remain within the scope of the disclosure.

DESCRIPTION OF EMBODIMENTS

Exemplary, non-limiting embodiments of a system and method for on-site detection of pathogens are disclosed hereinafter.

There is provided an on-site detection system for detecting pathogens in a liquid sample. The on-site detection system may comprise a filtration module,

an amplification module and a detection module. The liquid sample may be from various water sources such as natural/artificial bodies of water e.g. pond, reservoir, swimming pool, water playground, cooling tower, spa or the like. Advantageously, the on-site detection system may provide a cost-effective, on-site point-of-care, rapid test-to-result detection of pathogens in liquid samples with excellent sensitivity, so as to provide to provide an early warning system to complement existing laboratory culture methods.

The filtration module may be a filter device which employs dead-end and/or cross flow filtration technique to obtain/concentrate a sample population of pathogens from a liquid sample. In one embodiment, the filtration module employs a dead-end cross-flow hybrid filtration technique which comprises use of a combination of dead-end and cross-flow filtration elements. The filter device may comprise an inlet and a plurality of filtration elements e.g. a plurality of hollow fibre membranes in fluid communication with the inlet. The inlet of the filter device may be connected to a fluid extruding device e.g. syringe or pump to deliver a liquid sample. Each hollow fibre membrane may comprise a first end and a second end, an outer surface and an inner surface defining a lumen. The first end of the hollow fibre membrane may be open-ended and in fluid communication with the inlet of the filter device. The second end of the hollow fibre membrane may be blocked/restricted such that the liquid sample (i.e. feed) entering the filter device via the inlet is directed to pass through the pores of the plurality of hollow fibre membranes. In various embodiments, the filter device is configured such that liquid sample entering the lumen of the plurality of hollow fibre membranes flows in a direction from the first end to the second end, said direction being substantially tangential to the inner surface of the hollow fibre membrane. In some embodiments, as the liquid sample reaches the second end of the plurality of hollow fibre membranes, it encounters the restriction at the second end and liquid is forced to cross-flow through the pores on the plurality of hollow fibre membranes.

In various embodiments, the sample population of pathogen is retained/trapped in the lumen of the plurality of hollow fibre membranes after the liquid sample passes through the pores of the plurality of hollow fibre membranes. The sample population of pathogen may be collected/resuspended by flushing the plurality of hollow fibre membranes with a volume of resuspension fluid e.g. from about 0.2 ml to about 0.5 ml of phosphate-buffered saline or any other suitable solutions. The flushing of the plurality of hollow fibre membranes may be performed using a syringe detachably coupled to the inlet of the filter device and pushing/pulling the syringe plunger a few (e.g. 3-4 or more) times to dislodge the sample population of pathogen from the lumen of the plurality of hollow fibre membranes.

In various embodiments, the detection system may further comprise a pre-filter device to 'clean up' the liquid sample, for example, as part of the filtration module to remove debris which may be present in the liquid sample.

In various embodiments, in use, a syringe e.g. 100-ml syringe containing the liquid sample may be connected to the inlet of the filter device. The liquid sample may be introduced into the filter device via actuation of the syringe plunger. In various embodiments, particles above a predetermined cut-off size e.g. 0.2 microns are trapped inside the lumens of the plurality of hollow fibre membranes while liquid and particles below the cut-off size pass through the pores of the plurality of hollow fibre membranes. The 100-ml syringe containing the liquid sample may be disconnected from the filter device and a syringe e.g. 1-ml syringe containing 0.2 ml of resuspension fluid may be connected to the filter device. The resuspension fluid may be introduced into the lumens of the plurality of hollow fibre membranes to dislodge and collect the particles trapped therewithin. The aforementioned steps may be repeated more than one time to filter the desired volume of liquid sample.

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In various embodiments, a pump e.g. peristaltic pump with a suitable tubing may be connected to the inlet of the filter device. The liquid sample may

be introduced into the filter device via the actuation of the peristaltic pump's motor, under a suitable time setting.

5 The plurality of hollow fibre membranes may have an average pore diameter of about 0.05 μm , about 0.1 μm , about 0.2 μm , about 0.3 μm , about 0.4 μm , about 0.5 μm , about 0.6 μm , about 0.7 μm , about 0.8 μm , about 0.9 μm , or about 1 μm . In one exemplary embodiment, the plurality of hollow fibre membranes has an average pore diameter of about 0.2 μm .

10 The plurality of hollow fibre membranes may be made from synthetic polymers, cellulose, or synthetically modified cellulose. Synthetic polymers include, but are not limited to, polyethylene, polypropylene, polybutylene, poly (isobutylene), poly (methyl pentene), polysulfone, polyethersulfone, polyester, polyetherimide, polyacrylnitril, polyamide, polymethylmethacrylate (PMMA),
15 ethylenevinyl alcohol, and fluorinated polyolefins. In one exemplary embodiment, the plurality of hollow fibre membranes is made from polypropylene.

Advantageously, a filter device using a dead-end cross-flow hybrid filtration technique provides a relatively large surface area across which filtration
20 takes place (as compared to flat membrane). This may allow the filter device to filter increased volumes of water without a significant decrease in the filtration capability/capacity.

The on-site detection system may further comprise an amplification
25 module. The amplification module may be configured to amplify one or more target biological elements present in one or more target species of viable pathogens in the sample population of pathogens. The one or more target biological elements may be nucleic acids e.g. DNA or RNA.

30 In one exemplary embodiment, the amplification module may be a recombinase polymerase amplification (RPA) module. In some embodiments, part of the methods / processes for recombinase polymerase amplification

employ a mechanism that is familiar or known to those skilled in the art. The mechanism may be one that has been previously described in earlier documents e.g. US 7,270,981 B2. Amplifying DNA fragments may comprise the use of a recombinase for pairing oligonucleotide primers with homologous sequence in duplex DNA; a single-stranded DNA binding protein (SSB) for binding to displaced strands of DNA and prevent the primers from being displaced; and a strand-displacing polymerase for starting DNA synthesis where the primer has bound to the target DNA.

As will be appreciated, recombinase polymerase amplification may generally comprise the following steps: 1) a recombinase agent is contacted with a first and a second nucleic acid primer to form a first and a second nucleoprotein primer; 2) the first and second nucleoprotein primers are contacted to a double stranded target sequence to form a first double stranded structure at a first portion of said first strand and form a second double stranded structure at a second portion of said second strand so the 3' ends of said first nucleic acid primer and said second nucleic acid primer are oriented towards each other on a given template DNA molecule; 3) the 3' end of said first and second nucleoprotein primers are extended by DNA polymerases to generate first and second double stranded nucleic acids, and first and second displaced strands of nucleic acid; 4) the second and third steps are repeated until a desired degree of amplification is reached.

RPA may be used to detect DNA and RNA, preferably double stranded DNA but not limited to double stranded DNA as other nucleic acid molecules such as single stranded DNA or RNA can be turned into double stranded DNA using molecular biology techniques known in the art. The number of starting copies required for RPA may be less than about 10^4 copies, less than about 10^3 copies, less than about 10^2 copies or less than about 10 copies. RPA may amplify a target nucleic acid by at least 10 fold, at least 10^2 fold, at least 10^3 fold, at least 10^4 fold, at least 10^5 fold, at least 10^6 fold, or at least 10^7 fold.

The RPA module may be in the form of a kit for performing RPA. The kit may comprise one or more freeze-dried nucleic acid primers, dNTPs (deoxynucleotide triphosphates), one or more freeze-dried pellets of enzyme mixture, a rehydration buffer for reconstituting the pellets, 160-320 mM magnesium acetate solution, and positive control template DNA to test the activity of the kit components. As the reagents may be freeze-dried, it is possible to maintain the reagents as a dry formulation without the need for refrigeration to maintain the activity of the reagents. The kit may be easily transported and used at on-site locations.

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The RPA module may further comprise a heating device, said heating device comprising one or more incubator/heated/heater blocks which are capable of heating or maintaining the temperature of samples/reaction mixtures within a container placed thereon to/at a target/operating temperature. The one or more incubator/heated/heater blocks may allow processing of multiple samples/specimens concurrently. Each incubator/heated/heater block may be adapted to provide a heating temperature which is different from another incubator/heated/heater block. For example, one heater block may be configured to maintain a suitable temperature for lysing a test sample, for example, about 90°C to about 98°C, or about 92°C to about 96°C or about 93°C to about 95°C. Another heater block may be configured to maintain a suitable temperature for amplification to occur, for example, about 37°C to about 42°C, or about 39°C to about 40°C. The one or more incubator/heated/heater block may be configured to maintain a substantially constant temperature, e.g. 95°C throughout the duration of lysing and 37°C throughout the duration of amplification. The heating device may be portable and battery-operated such that it is suitable for use in on-site/out-field testing. In various embodiments, the RPA module may comprise a plurality of heating devices.

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In order to amplify a target nucleic acid sequence belonging to a target pathogen, primers may be designed to anneal to a complementary target nucleic acid molecule by nucleic acid hybridization. A primer may be extended along the

target nucleic acid molecule by a polymerase enzyme. Accordingly, primers may be used to amplify a target nucleic acid molecule, wherein the sequence of the primer is specific for the target nucleic acid molecule, for example so that the primer will hybridize to the target nucleic acid molecule under stringent
5 hybridization conditions.

In various embodiments, the RPA module may employ a two-step amplification process where a probe and a pair of primers are used to perform amplification. The second additional step may advantageously improve the
10 stringency of the amplification reaction. In the first step, a pair of primers may be used to amplify a region on the target nucleic acid molecule, resulting in the formation of primary amplicons between about 300 to about 400 base pairs (bp). In the second step, a probe may be used to bind to complementary sequences on the primary amplicons, and upon removal of a 3'-blocker on the probe, the
15 probe now acts as a forward primer, resulting in amplification to form secondary amplicons between about 100 to about 200 bp. In various embodiments, secondary amplicons may be derived from primary amplicons. It will be appreciated that the RPA module may use a one-step amplification process where a probe and at least one primer is used to perform amplification. For
20 example, a probe (acting as a forward primer) and a reverse primer may be used to amplify a region on the target nucleic acid molecule.

In general, the specificity of a primer increases with its length. For example, a primer that has a length of 30 nucleotides will anneal to a target
25 sequence with a higher specificity than another primer that has only 15 nucleotides in length. Thus, to obtain greater specificity, probes and primers may be designed and selected to have a length of at least about 15, at least about 20, at least about 25, at least about 30, at least about 35, at least about 40, at least about 45, or at least about 50 or more nucleotides.

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In one exemplary embodiment, the amplification module may be capable of amplifying DNA fragments corresponding to *Pseudomonas aeruginosa*. The

amplification module may be capable of amplifying nucleic acid sequences in *Pseudomonas aeruginosa* algD gene for GDP-mannose dehydrogenase (GenBank Accession No. Y00337.1; GI No. 45267) using primers and probes comprising nucleotide sequences having at least 80% sequence identity to a

5 nucleotide sequence selected from the group consisting of SEQ ID NO. 1 to SEQ ID NO. 5. An exemplary nucleic acid sequence that encodes *Pseudomonas aeruginosa* algD gene for GDP-mannose dehydrogenase is provided in SEQ ID NO. 16 of the sequence listing. In one example, a forward primer based on or comprising a nucleotide sequence of SEQ ID NO.1, a reverse primer based on

10 or comprising a nucleotide sequence of SEQ ID NO. 2 and a lateral flow probe based on or comprising a nucleotide sequence of SEQ ID NO. 4 may be used to amplify target nucleic acid sequences for subsequent detection using a lateral flow system. In another example, a forward primer based on or comprising a nucleotide sequence of SEQ ID NO. 1, a reverse primer based on or comprising

15 a nucleotide sequence of SEQ ID NO. 3 and a fluorescence labelled probe based on or comprising a nucleotide sequence of SEQ ID NO. 5 may be used to amplify target nucleic acid sequences for subsequent detection using a fluorescence detection system. *Pseudomonas aeruginosa* is a gram-negative, rod-shaped, asporogenous, and monoflagellated bacterium which can be found in

20 environments such as soil, water, humans, animals, plants, sewage, and hospitals. *Pseudomonas aeruginosa* rarely causes disease in healthy humans. It is usually linked with patients whose immune system is compromised by diseases or trauma. It gains access to these patients' tissues through the burns, for the burn victims, or through an underlying disease, like cystic fibrosis.

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In another exemplary embodiment, the amplification module may be capable of amplifying DNA fragments corresponding to *Legionella pneumophila*. The amplification module may be capable of amplifying nucleic acid sequences in *Legionella pneumophila* 16S ribosomal RNA (GenBank Accession No.

30 M59157.1; GI No: 175168) using primers and probes comprising nucleotide sequences having at least 80% sequence identity to a nucleotide sequence selected from the group consisting of SEQ ID NO. 6 to SEQ ID NO. 10. An

exemplary nucleic acid sequence that encodes *Legionella pneumophila* 16S ribosomal RNA is provided in SEQ ID NO. 17 of the sequence listing. In one example, a forward primer based on or comprising a nucleotide sequence of SEQ ID NO. 6, a reverse primer based on or comprising a nucleotide sequence of SEQ ID NO. 7 and a lateral flow probe based on or comprising a nucleotide sequence of SEQ ID NO. 9 may be used to amplify target nucleic acid sequences for subsequent detection using a lateral flow system. In another example, a forward primer based on or comprising a nucleotide sequence of SEQ ID NO. 6, a reverse primer based on or comprising a nucleotide sequence of SEQ ID NO. 8 and a fluorescence labelled probe based on or comprising a nucleotide sequence of SEQ ID NO. 10 may be used to amplify target nucleic acid sequences for subsequent detection using a fluorescence detection system. *Legionella pneumophila* is a thin, aerobic, pleomorphic, flagellated, nonspore-forming, Gram-negative bacterium of the genus *Legionella*. *Legionella pneumophila* is the primary human pathogenic bacterium in this group and is the causative agent of Legionnaires' disease, also known as legionellosis.

In another exemplary embodiment, the amplification module may be capable of amplifying DNA fragments corresponding to *Enterococcus species* e.g. *Enterococcus faecium*. The amplification module may be capable of amplifying nucleic acid sequences in *Enterococcus faecium* strain (ATCC 19434) 16S ribosomal RNA gene, partial sequence (GenBank Accession No. DQ411813.1; GI No. 89357459), using primers and probes comprising nucleotide sequences having at least 80% sequence identity to a nucleotide sequence selected from the group consisting of SEQ ID NO. 11 to SEQ ID NO. 15. An exemplary nucleic acid sequence that encodes *Enterococcus faecium* strain (ATCC 19434) 16S ribosomal RNA gene, partial sequence is provided in SEQ ID NO. 18 of the sequence listing. In one example, a forward primer based on or comprising a nucleotide sequence of SEQ ID NO. 11, a reverse primer based on or comprising a nucleotide sequence of SEQ ID NO. 12 and a lateral flow probe based on or comprising a nucleotide sequence of SEQ ID NO. 14 may be used to amplify target nucleic acid sequences for subsequent detection using a lateral

flow system. In another example, a forward primer based on or comprising a nucleotide sequence of SEQ ID NO. 11, a reverse primer based on or comprising a nucleotide sequence of SEQ ID NO. 13 and a fluorescence labelled probe based on or comprising a nucleotide sequence of SEQ ID NO. 15 may be used to amplify target nucleic acid sequences for subsequent detection using a fluorescence detection system. *Enterococcus faecium* is a Gram-positive, alpha-hemolytic or nonhemolytic bacterium in the genus *Enterococcus*. It can be commensal (innocuous, coexisting organism) in the gastrointestinal tract of humans and animals, but it may also be pathogenic, causing diseases such as neonatal meningitis or endocarditis.

Table 1 provides a list of exemplary nucleic acid sequences of primers and probes that may be used in a RPA assay in an exemplary embodiment. Each of primer/probe sequence numbers 1 to 15 may be derived from SEQ ID NO. 1 to SEQ ID NO. 15 of the sequence listing, respectively. Primer/probe sequences 1 to 5 may be used for amplifying nucleic acid sequences in *Pseudomonas aeruginosa* algD gene for GDP-mannose dehydrogenase (GenBank Accession No. Y00337.1; GI No. 45267). Primer/probe sequences 6 to 10 may be used for amplifying nucleic acid sequences in *Legionella pneumophila* 16S ribosomal RNA (GenBank Accession No. M59157.1; GI No: 175168). Primer/probe sequences 11 to 15 may be used for amplifying nucleic acid sequences in *Enterococcus faecium* strain (ATCC 19434) 16S ribosomal RNA gene, partial sequence (GenBank Accession No. DQ411813.1; GI No. 89357459).

Primer sequences 1, 6 and 11 may be used as forward primers in the RPA assay. As shown in Table 1, primer sequences 1, 6 and 11 are identical to SEQ ID NO. 1, 6 and 11 of the sequence listing, respectively. Primer sequences 2, 7 and 12 may be used as reverse primers in the RPA assay for amplifying products which will be detected using a lateral flow assay. As shown in Table 1, the reverse primers (lateral flow) are biotinylated at the 5' end of the primer sequences. Primer sequences 3, 8 and 13 may be used as reverse primers in the RPA assay for amplifying products which will be detected using fluorescence detection. As

shown in Table 1, primer sequences 3, 8 and 13 are identical to SEQ ID NO. 3, 8 and 13 of the sequence listing, respectively. Probe sequences 4, 9 and 14 may be used as probes in the RPA assay for amplifying products which will be detected using a lateral flow assay. As shown in Table 1, probe sequences 4, 9 and 14 are labelled with either FAM (a type of fluorescein dye) or 5'-DIG (digoxigenin) in alternative sequences. Probe sequences 5, 10 and 15 may be used as probes in the RPA assay for amplifying products which will be detected using fluorescence detection. As shown in Table 1, a dSpacer is positioned between a Black-hole quencher (BHQ) and a fluorophore e.g. iFluor within the probe sequences 5, 10 and 15. The BHQ quenches the fluorophore when in close proximity. Cleavage of the dSpacer separates the BHQ from the fluorophore. In the exemplary embodiment, primers and probes for detecting different bacteria e.g. *Pseudomonas aeruginosa*, *Legionella pneumophila*, *Enterococcus faecium* may be mixed together in the same reaction mixture to allow multiplex detection of different target bacteria within a water sample.

Table 1. Exemplary nucleic acid sequences of primers and probes that may be used in RPA assay (labelled and non-labelled)

Primer/ Probe Sequence No.	Nucleotide sequence	FUNCTION
1	5'- TTCCCTCGCAGAGAAAACATCCTATCACCG CG-3'	Forward Primer
2	5'- /5Biosg/GGGCGACTTGCCCTGGTTGATCAG GTCGATCT-3'	Reverse Primer (Lateral Flow)

3	5'- GGGCGACTTGCCCTGGTTGATCAGGTCGA TCT-3'	Reverse Primer (Fluorescence)
4	5'/56FAM/CGGCCACCTCATTAACGGCGCGA CAAACAATC/idSp/AGGTGAATGCGATGC/3P hos/-3' Alternative sequence 5'/5DigN/CGGCCACCTCATTAACGGCGCGA CAAACAATC/idSp/AGGTGAATGCGATGC/3P hos/-3'	Probe (Lateral Flow)
5	5'- CGGCCACCTCATTAACGGCGCGACAAACA ATCGAGG/iFluorT/G/idSp/A/iBHQ- 1dT/GCGATGCGAATCAG/3Phos/-3'	Probe (Fluorescence)
6	5'- AGGGTTGATAGGTTAAGAGCTGATTAAGT GA-3'	Forward Primer
7	5'- /5Biosg/ATATAACCAACAGCTAGTTGACATC GTTTACA-3'	Reverse Primer (Lateral Flow)
8	5'- ATATAACCAACAGCTAGTTGACATCGTTTA CA-3'	Reverse Primer (Fluorescence)
9	5'/5DigN/GGAGAGGGTAGTGGAATTTCCGG TGTAGCGGT/idSp/AAATGCGTAGAGATCG/3 Phos/-3' Alternative sequence	Probe (Lateral Flow)

	5'/56FAM/GGAGAGGGTAGTGGAATTTCCGG TGTAGCGGT/idSp/AAATGCGTAGAGATCG/3 Phos/-3'	
10	5'- GGAGAGGGTAGTGGAATTTCCGGTGTAGC GGTGAAG/iFluorT/GC/idSp/iBHQ- 1dT/AGAGATCGGAAGGA/3Phos/-3'	Probe (Fluorescence)
11	5'- TGCATTAGCTAGTTGGTGAGGTAACGGCTC ACCA-3'	Forward Primer
12	5'- /5Biosg/CTTTCACATCAGACTTAAGAAACCG CCTGCGC-3'	Reverse Primer (Lateral Flow)
13	5'- CTTTCACATCAGACTTAAGAAACCGCCTGC GC-3'	Reverse Primer (Fluorescence)
14	5'/5DigN/AAACTCTGTTGTTAGAGAAGAAC AAGGATGA/idSp/AGTAACTGTTCATCC/3Pho s/-3' Alternative sequence 5'/56FAM/AAACTCTGTTGTTAGAGAAGAAC AAGGATGA/idSp/AGTAACTGTTCATCC/3Pho s/-3'	Probe (Lateral Flow)
15	5'- ACTCTGTTGTTAGAGAAGAACAAGGATGAG AG/iFluorT/A/idSp/C/iBHQ- 1dT/GTTCATCCTTGACG/3Phos/-3'	Probe (Fluorescence)

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The primers/probes may have nucleic acid sequences with at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 99% or about 100% sequence identity to the nucleic acid sequences selected from the group consisting of SEQ ID NO. 1 to SEQ ID NO. 15.

In order to monitor the amplification of the target nucleic acids in the RPA reaction, detectable labels may be used and these include but are not limited to enzymes, enzyme substrates, coenzymes, enzyme inhibitors, fluorescent markers, chromophore, luminescent markers, radioisotopes (including radionucleotides) and one member of the primer pair. Specific examples may include but are not limited to fluorescein, phycobiliprotein, tetraethyl, rhodamine, and beta-gel. Bind pairs may include but are not limited to biotin-avidin, biotin-streptavidin, antigen-antibody, ligand receptor, and analogs and mutants of the binding primer pairs.

In one embodiment, detection probes can be used. The detection probe may be a third oligonucleotide primer which recognises the target amplicon and is typically homologous to sequences between the main amplification primers. The detection probe may be about 46-52 oligonucleotides long and may comprise a label e.g. 5'-FAM (a type of fluorescein dye) or 5'-DIG (digoxigenin) or other suitable dyes/labels; an abasic site e.g. internal dSpacer or THF (tetrahydrofuran); and a 3'-blocking group. In various embodiments, prior to annealing to target sequence, the detection probe is blocked from polymerase extension by the 3'-blocking group by making the last nucleotide a dideoxy nucleotide or a 3' phosphate group. In various embodiments, the dSpacer is typically positioned about 30 bases from the 5' end of the detection probe and about 16 bases from the 3' end. In various embodiments, after the detection probe has annealed to the target sequence, an endonuclease IV (Nfo) and/or exonuclease III (exoIII) nuclease recognises and cleaves the dSpacer to remove the 3' end extension blocker (i.e. dideoxynucleotide or 3'-phosphate), thus

allowing the 3' end of the detection probe to act as a primer. In various embodiments, the opposing amplification primer is typically labelled with another label such as biotin. In various embodiments, as the amplification reaction progresses, amplified products/ amplicons which are labelled with both FAM and
5 biotin would form.

In another embodiment, in the case of fluorescence detection, a dSpacer is inserted between a Black-hole quencher (BHQ) and a fluorophore within a probe. The BHQ quenches the fluorophore when in close proximity. Cleavage of
10 the dSpacer separates the BHQ from the fluorophore. As a result, the fluorophore is released and separated from the BHQ, leading to an increase in fluorescence.

It may be appreciated that the position of the detection probe relative to its corresponding primers may be chosen to avoid the possibility of the primer
15 artefacts being detected by the detection probe.

In use, the RPA reaction may be performed within a reaction tube. A freeze-dried pellet containing an enzyme mixture may be reconstituted with the rehydration buffer, the first and second nucleic acid primers, and the target
20 nucleic acid and water to a desired volume. Magnesium acetate solution may be added to initiate the reaction. The reaction tube containing the reaction mixture may be placed on an incubator block and heated for a period of time until the target nucleic acid achieves a desired degree of amplification.

25 Recombinase polymerase amplification may be performed at a temperature of between about 20°C to about 50°C, between about 24°C to about 46°C, between about 28°C to about 42°C, between about 32°C to about 38°C, or between about 36°C to about 38°C. In one exemplary embodiment, recombinase polymerase amplification may be performed between about 37°C to about 42°C.

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The amplification module may have a sensitivity of at least about 80%, at least about 85%, at least about 90%, at least about 95% or at least about 99%.

The amplification module may have a specificity of at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95%.

Advantageously, RPA may provide a fast, portable detection assay which is suited for field and on-site testing. In various embodiments, RPA works optimally at a temperature of around 37-42°C and may be capable of amplifying nucleic acid molecules to detectable levels in about 3-10 minutes. In various embodiments, RPA does not require thermal or chemical melting, and therefore obviates the need for costly equipment such as a thermocycler. This is in contrast to PCR which relies on multiple cycles of thermal melting (denaturing) at high temperatures followed by hybridization and elongation at a reduced temperature. Complex temperature control of multiple reactions is required in PCR and this necessitates the use of a thermocycler. In addition, RPA may also be capable of multiplexing by combining multiple primers in the same reaction tube to amplify more than one target nucleic acids belonging to one or more pathogens. It will be appreciated that in various embodiments, the amplification module does not utilise PCR that requires thermal cycling.

The on-site detection system may further comprise a detection module. The detection module may be configured to detect presence of amplified products of the one or more target biological elements present in the one or more target species of viable pathogens. In one exemplary embodiment, the detection module may comprise a lateral flow strip configured to detect the presence of amplified products. The lateral flow strip may be used in a qualitative manner to give positive/negative answer corresponding to the presence or absence of analyte in a test sample. The lateral flow strip may be incorporated into a lateral flow device provided with a receiving port for loading the test sample and at least one transparent window at the signal zone, thus providing a simple self-contained detecting device.

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In general, lateral flow strips may be based on a series of capillary beds which have the ability to transport fluid spontaneously, such as pieces of porous

paper, sintered polymer or the like. In various embodiments, lateral flow strips may comprise a sample pad which can act as a sponge and holds an excess of sample fluid. In general, once soaked, the fluid may migrate to a conjugate pad in which the manufacturer has stored the so-called conjugate, a dried format of
5 bio-active particles or probes in a matrix that contains everything to provide an optimised reaction between the target molecule and its binding partner that can be immobilised on or to a surface. In various embodiments, as the test sample fluid dissolves the matrix, it also dissolves the particles and in one combined transport action the sample and conjugate mix while flowing through the porous
10 structure. In this way, the analyte may bind to the particles while migrating further through an additional capillary bed. This material may have one or more areas (often called stripes) where a third molecule has been immobilised. In various embodiments, by the time the sample-conjugate mix reaches these strips, analyte has been bound on the particle and the third 'capture' molecule binds the
15 complex. After a while, when more and more fluid has passed the stripes, particles may accumulate and the area changes color. In various embodiments, after passing these reaction zones the fluid enters the final porous material, the wick, that simply acts as a waste container.

20 In one exemplary embodiment, the use of lateral flow to detect DNA amplification may be based on dual labeled probes and gold nanoparticles. It will be appreciated that any other suitable particles e.g. carbon nanoparticles may also be used in place of gold nanoparticles. In various embodiments, in use, to detect the presence of a double labelled amplicon, a pad may be incorporated
25 with visible (nano-colloidal) gold particles coupled to an antibody which recognises one of the two labels on the amplicon. A test sample which may contain double labelled amplicons e.g. Biotin/FAM-amplicons may be loaded onto the pad and the double labelled amplicon may form a complex with the gold particles. The complexes may then travel along the lateral flow strip and reaches
30 a test/detection lane/line where capture molecules e.g. streptavidin or immobilised anti-biotin antibodies may be disposed thereon. The capture molecule may be configured to bind to one of the labels e.g. biotin on the double

labelled amplicon. Where a double labelled amplicon is present, a visible line will appear on the test lane due to the capture of the complexes formed by the double labelled amplicon and the gold particles. Where a double labelled amplicon is absent, a visible line will not appear on the test lane, indicating the absence of a double labelled amplicon.

This above approach of using dual labeled amplicons advantageously allows multiplex detection in the same samples.

In another exemplary embodiment, more than one lateral flow kits may be used in conjunction with a test sample which has undergone a sequence of dilutions to provide detection results in a simplified Most Probable Number (MPN) method/format. The simplified MPN method disclosed herein may be considered as an adaptation of a standard MPN method but is unlike the standard MPN method where statistical analysis is carried out. The simplified MPN method may provide an estimated range of quantity of bacteria in the original (i.e. undiluted) sample by carrying out serial dilution of the original sample. The simplified MPN method works based on the assumptions that (1) the sample can be diluted to a point where no target DNA/bacteria can be detected; and (2) the corresponding dilution which gives a positive result on the lateral flow kit means that there is at least one or more than one molecule/entity/CFU/cell of the target. With the dilution factors and results from the lateral flow kits, it may be possible to calculate the estimated amount of bacteria in the original (i.e. undiluted) sample. For example, if a 10^{-3} sample (i.e. 1:1000 dilution) contains at least 1 cell, then a 10^{-2} sample would contain at least 10 cells, and a 10^{-1} sample would contain at least 100 cells, and the undiluted sample would contain at least 1000 cells.

In another exemplary embodiment, the detection module may comprise a fluorescence detector for detecting the presence of fluorescence-labelled amplified products. The fluorescence detector may comprise a housing for accommodating a test sample e.g. a vial/tube containing the test sample, a light source with a suitable wavelength for projecting light towards the test sample,

and a photodiode/photomultiplier which converts light into an electrical current/signal. The housing may be substantially opaque to prevent light from external sources from reaching the test sample and interfering with the detection of fluorescence-labelled amplified products. The light source may be used to
5 excite the fluorescence probe to generate fluorescent light which may be detected by the photodiode/photomultiplier. In various embodiments, the fluorescence detector may be portable and suitable for on-site detection.

The on-site detection system may further comprise a treatment module.
10 The treatment module may comprise a composition for preferentially interacting with one or more biological elements present in non-viable pathogens in the sample population of pathogens to render said one or more biological elements incapable of amplification. In various embodiments, the treatment module may be optional.

15 One way that may be used to distinguish between viable and non-viable (i.e. live and dead) pathogens is through the integrity of the membrane. For example, viable pathogens may have intact cell membranes which are able to exclude substances from entering the cell. On the other hand, non-viable
20 pathogens may have compromised cell membranes which are unable to exclude substances from entering the cell.

Treating a sample population of pathogens with the composition of the treatment module may allow a user to isolate viable pathogens from a sample
25 population of pathogens comprising both viable and non-viable pathogens. Advantageously, the treatment module may allow subsequent testing and analysis, e.g. DNA amplification to be limited to viable pathogens, as non-viable pathogens are rendered incapable of amplification.

30 The composition for preferentially interacting with one or more biological elements may comprise phenanthridium derivatives which include but are not limited to propidium monoazide (PMA) and ethidium monoazide (EMA). The one

or more biological elements may include but are not limited to DNA and RNA. The interaction of the composition with the one or more biological elements may be chemical in nature, e.g. covalent binding/crosslinking of the composition with the biological element and the like. In one exemplary embodiment, the phenanthridium derivative may be propidium monoazide. Propidium monoazide may modify nucleic acid e.g. DNA, thereby interfering with the DNA's ability to undergo amplification. Advantageously, use of propidium monoazide may provide a reliable and convenient way to perform treatment of a sample with increased selectivity. Propidium monoazide may be used on a wide range of bacterial species, penetrating only into dead bacterial cells with compromised membrane integrity but not into live cells with intact cell membranes/cell walls.

In use, a sample population of pathogens may be mixed with a composition comprising propidium monoazide. The mixture may be first incubated in the dark for a period of time e.g. about 10 minutes; and thereafter may be incubated on a photolysis device (i.e. exposing the mixture to a light source for DNA binding and inactivation of free unbound PMA). The concentration of PMA may be from about 10 μ M to about 100 μ M. The period of time for incubation may be from about 1 minutes to about 60 minutes.

Non-limiting examples of pathogens which may be detected with the on-site detection system include but are not limited to: *B. pertussis*, *Leptospira pomona*, *S. paratyphi* A and B, *C. diphtheriae*, *C. tetani*, *C. botulinum*, *C. perfringens*, *C. fescer* and other gas gangrene bacteria, *B. anthracis*, *P. pestis*, *P. multocida*, *Neisseria meningitidis*, *N. gonorrhoeae*, *Hemophilus influenzae*, *Actinomyces* (e.g., *Nocardia*), *Acinetobacter*, *Bacillaceae* (e.g., *Bacillus anthracis*), *Bacteroides* (e.g., *Bacteroides fragilis*), *Blastomycosis*, *Bordetella*, *Borrelia* (e.g., *Borrelia burgdorferi*), *Brucella*, *Campylobacter*, *Chlamydia*, *Coccidioides*, *Corynebacterium* (e.g., *Corynebacterium diphtheriae*), *E. coli* (e.g., Enterotoxigenic *E. coli* and Enterohemorrhagic *E. coli*), *Enterobacter* (e.g. *Enterobacter aerogenes*), *Enterobacteriaceae* (*Klebsiella*, *Salmonella* (e.g., *Salmonella typhi*, *Salmonella enteritidis*, *Serratia*, *Yersinia*, *Shigella*),

Enterococcus (e.g. *Enterococcus faecium*), *Erysipelothrix*, *Haemophilus* (e.g., *Haemophilus influenza* type B), *Helicobacter*, *Legionella* (e.g., *Legionella pneumophila*), *Leptospira*, *Listeria* (e.g., *Listeria monocytogenes*), *Mycoplasma*, *Mycobacterium* (e.g., *Mycobacterium leprae* and *Mycobacterium tuberculosis*),
 5 *Vibrio* (e.g., *Vibrio cholerae*), Pasteurellaceae, *Proteus*, *Pseudomonas* (e.g., *Pseudomonas aeruginosa*), Rickettsiaceae, Spirochetes (e.g., *Treponema* spp., *Leptospira* spp., *Borrelia* spp.), *Shigella* spp., *Meningioccus*, *Pneumococcus* and *Streptococcus* (e.g., *Streptococcus pneumoniae* and Groups A, B, and C Streptococci), Ureaplasmas. *Treponema pallidum*, *Staphylococcus aureus*,
 10 *Pasteurella haemolytica*, *Corynebacterium diphtheriae* toxoid, Meningococcal polysaccharide, *Bordetella pertussis*, *Streptococcus pneumoniae*, *Clostridium tetani* toxoid, and *Mycobacterium bovis*.

There is also provided an on-site detection method for detecting
 15 pathogens in a liquid sample using the on-site detection system as disclosed herein.

BRIEF DESCRIPTION OF FIGURES

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Fig. 1 is a schematic diagram showing collection and concentration of a sample population of pathogens e.g. bacteria in an exemplary embodiment.

Fig. 2 is a schematic diagram showing treatment of a sample population
 25 of bacteria with propidium monoazide (PMA) in an exemplary embodiment.

Fig. 3 is a schematic diagram showing recombinase polymerase amplification of a test sample containing a sample population of bacteria in an exemplary embodiment.

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Fig. 4A is a schematic diagram showing detection of amplified products of one or more bacteria of interest using lateral flow devices in an exemplary embodiment.

5 Fig. 4B is a schematic diagram showing detection of amplified products of one or more bacteria of interest using a fluorescence detector in an exemplary embodiment.

10 Fig. 5 is a schematic diagram showing an interpretation of test results displayed on lateral flow strips in an exemplary embodiment.

Fig. 6 is a schematic diagram showing the amplification process of a primary amplicon/amplified product in an exemplary embodiment.

15 Fig. 7 is a schematic diagram showing a lateral flow strip in an exemplary embodiment.

20 Fig. 8 is a schematic diagram showing a complex formed between an amplified DNA product and a nanocolloidal gold particle in an exemplary embodiment.

Fig. 9 is a schematic diagram showing a test lane segment of a lateral flow strip in an exemplary embodiment.

25 Fig. 10 is a schematic diagram showing the control lane segment of a lateral flow strip in an exemplary embodiment.

30 Fig. 11 is a photograph of lateral flow strip test results for detection of cells treated/untreated with propidium monoazide (PMA) in an exemplary embodiment.

Fig. 12 is a graph showing relative fluorescence unit (RFU) against number of amplification cycles of target DNA using qPCR in an exemplary embodiment.

5 Fig. 13 is a graph showing melt curve analysis of the qPCR products in an exemplary embodiment.

Fig. 14 is a graph showing melt peak analysis of the qPCR products in an exemplary embodiment.

10 Fig. 15 is a flowchart showing a method of performing a RPA-MPN experiment in an exemplary embodiment.

Fig. 16 is a photograph of lateral flow strip test results for the RPA-MPN experiment in an exemplary embodiment.

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Fig. 17 is a photograph of spread plates for the RPA-MPN experiment in an exemplary embodiment.

20 Fig. 18 is a graph showing relative fluorescence unit (RFU) against time in a real-time DNA amplification in an exemplary embodiment.

Fig. 19 is a graph showing relative fluorescence unit (RFU) against time in a real-time DNA amplification of samples with different initial concentrations of a DNA template in an exemplary embodiment.

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Fig. 20 is a graph showing Log CFU (*E. faecium*) against fluorescence signal (threshold point) in an exemplary embodiment.

30 Fig. 21 is a partial nucleic acid sequence of *Pseudomonas aeruginosa* algD gene for GDP-mannose dehydrogenase at positions 121 to 660 in an exemplary embodiment.

Fig. 22 is a partial nucleic acid sequence of *Legionella pneumophila* 16S ribosomal RNA at positions 421 to 840 in an exemplary embodiment.

Fig. 23 is a partial nucleic acid sequence of *Enterococcus faecium* strain (ATCC 19434) 16S ribosomal RNA gene at positions 181 to 600 in an exemplary embodiment.

DETAILED DESCRIPTION OF FIGURES

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Example embodiments of the disclosure will be better understood and readily apparent to one of ordinary skill in the art from the following discussions and if applicable, in conjunction with the figures. It should be appreciated that other modifications related to structural, and material changes may be made without deviating from the scope of the disclosure. Example embodiments are not necessarily mutually exclusive as some may be combined with one or more embodiments to form new exemplary embodiments.

Figs. 1 to 10 describe a method of on-site detection of pathogen using an on-site detection system as described herein.

Fig. 1 is a schematic diagram showing collection and concentration of a sample population of pathogens e.g. bacteria 100 in an exemplary embodiment. A test sample 102 of water containing bacteria 100 is collected in a container e.g. beaker 104. A 100-ml syringe 106 is used to draw a volume of the test sample 102. The syringe 106 is coupled to an inlet 108 of a dead-end cross-flow hybrid filter 110. The filter 110 further comprises hollow fibre membranes 112 configured to provide dead-end cross-flow filtration and an outlet 114 for allowing filtered waste water to exit from the filter 110 and is collected in a container 116. The plunger of the 100-ml syringe 106 is actuated to cause the test sample 102 to filter through the filter 110. As the test sample 102 flows through the filter 110, the bacteria 100 is trapped and concentrated within the hollow fibre membranes

112. The 100-ml syringe is then removed and the filter 110 is connected to a 1-ml syringe 118 containing 0.2 ml of phosphate-buffered saline (PBS) solution. It will be appreciated that any other suitable solution may also be used in place of PBS solution. The PBS solution is injected/ flushed into the filter 110 to resuspend
 5 the trapped bacteria 100 in the PBS solution. The step of flushing the filter 110 may be repeated 3-4 times by pushing and pulling (indicated by reference numeral 120) the plunger of the 1-ml syringe 118 to ensure that the bacteria 100 is dislodged from the hollow fibre membranes 112 and resuspended in the PBS solution. The steps of passing the test sample 102 through the filter 110 and
 10 flushing the filter 110 with PBS solution may be repeated if the test sample 102 is more than 100 ml.

Fig. 2 is a schematic diagram showing treatment of a sample population of bacteria 200 with propidium monoazide (PMA) in an exemplary embodiment.
 15 **PMA is a DNA cross-linker which modifies free floating DNA or DNA in dead/ non-viable bacteria (with compromised cell integrity), preventing their amplification during amplification assays such as polymerase chain reaction or recombinase polymerase amplification. PMA does not affect DNA in live cells and hence will not affect their amplification and detection. It would be appreciated that treatment**
 20 of the sample population of bacteria with PMA is an optional step.

The sample population of bacteria 200 containing both viable and non-viable bacteria is suspended in PBS solution within a syringe 202. 0.1 ml of the sample population of bacteria 200 is each transferred to a first tube 204 and a
 25 second tube 206. The first tube 204 is treated with a PMA composition while the second tube 206 is not treated with the PMA composition. Both the first tube 204 and second tube 206 are placed in a substantially opaque box 208 and incubated in the dark for 10 minutes. Thereafter, the first tube 204 and second tube 206 are placed on a photolysis device 210 and incubated for 10 minutes. At the end of
 30 the photolysis treatment, the sample population of bacteria 200 in the first tube 204 has been modified such that DNA of non-viable bacteria in the sample population of bacteria 200 are bound to PMA and are incapable of being amplified

in an amplification reaction e.g. polymerase chain reaction or recombinase polymerase amplification. The sample population of bacteria 200 in the second tube 206 has not been exposed to PMA and therefore DNA of non-viable bacteria in the sample population of bacteria 200 are still capable of being amplified in an amplification reaction. The sample population of bacteria from the first tube 204 and second tube 206 are drawn into a first syringe 214 and a second syringe 216 respectively for further processing.

Fig. 3 is a schematic diagram showing recombinase polymerase amplification of a test sample 300 containing a sample population of bacteria in an exemplary embodiment. The recombinase polymerase amplification (RPA) is used to amplify DNA belonging to one or more target bacteria of interest. The test sample 300 is contained within a syringe 302 and placed on a heater block 304 to be heated at 95°C for 10 minutes to lyse the test sample 300. Excess test sample is pushed out into a tube 306 such that 50 µL of the test sample 300 remains in the syringe 302. Next, a reaction premix solution 308 is prepared from a RPA kit 314 by reconstituting the freeze-dried reaction premix with rehydration buffer, magnesium acetate solution and PCR-grade water. The reaction premix solution 308 also contains primer and probe sequences designed to bind to the DNA of the one or more bacteria of interest. A blank sample 310 and a positive control sample 312 are also provided in the RPA kit 314. The test sample 300, blank sample 310 and positive control sample 312 are each aspirated with their respective reaction premix solution 308 by pushing and pulling the plungers of the syringes 3-4 times to mix the samples with the reaction premix solutions. The test sample 300, blank sample 310 and positive control sample 312, each mixed with the reaction premix solution, are placed on a heater block 316 to be heated at 37°C for 25 minutes to amplify DNA of the one or more bacteria of interest. At the end of the amplification procedure, if the test sample 300 contains the one or more bacteria of interest, the test sample 300 would contain amplified products thereof. Similarly, the positive control sample 312 would also contain amplified products of the one or more bacteria of interest. The blank sample 310 should not contain amplified products of the one or more bacteria of interest.

Fig. 4A is a schematic diagram showing detection of amplified products of one or more bacteria of interest using lateral flow devices in an exemplary embodiment. A test sample 400, a blank sample 402 and a positive control sample 404 are first diluted with a dilution buffer 406. 0.1 ml of the test sample 400, blank sample 402 and positive control sample 404 are each loaded into lateral flow devices 410, 412 and 414 respectively. The test sample 400, blank sample 402 and 404 are allowed to flow along the lateral flow strips via capillary action. For example, the lateral flow device 410 comprises a loading port 416 and a substantially transparent window 418 exposing the signal zone. The signal zone may comprise a control lane and at least one test lane (not shown). Appearance of a visible line on the test lane indicates the presence of an analyte of interest, i.e. amplified products of the one or more bacteria of interest. Appearance of a visible line on the control lane indicates that the test has run correctly.

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Fig. 4B is a schematic diagram showing detection of amplified products of one or more bacteria of interest using a fluorescence detector 426 in an exemplary embodiment. A test sample 420, blank sample 422 and positive control sample 424, each mixed with a RPA reaction premix solution, are placed on a heated block to be heated at 37°C for 15 minutes. The reaction is then monitored with a LED-sourced fluorescence detector 426.

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Fig. 5 is a schematic diagram showing an interpretation of test results displayed on lateral flow strips in an exemplary embodiment. The lateral flow strip test is used to detect the presence of amplified products belong to one or more bacteria of interest. The lateral flow strip comprises a first lane 1, a second lane 2 and a control lane C. A line/band appears on the first lane 1 if amplified products belonging to a first bacteria A is present. A line appears on the second lane 2 if amplified products belonging to a second bacteria B is present. A line on control lane C is used to confirm that the test has run correctly and will appear whether or not amplified products belonging to the bacteria of interest are present. Absence of a line on control lane C indicates that there is an error in the test. In

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the exemplary embodiment, strip 500 displays a line on control lane C only, indicating that the first bacteria A and second bacteria B are absent. Strip 502 displays a line on control line C and lane 1, indicating that the first bacteria A is present. Strip 504 displays a line on control lane C and lane 2, indicating that the second bacteria B is present. Strip 506 displays a line on control lane C, lane 1 and lane 2, indicating that the first bacteria A and the second bacteria B are present. Strip 508 does not display lines on control lane C, lane 1 and lane 2, indicating that there is an error and no results can be obtained.

Fig. 6 is a schematic diagram showing the amplification process 600 of a primary amplicon/amplified product 602 in an exemplary embodiment. A probe labelled with a first label e.g. FAM-labelled probe 604 and a primer labelled with a second label e.g. biotin-labelled primer 606 capable of selectively binding to a specified target nucleic acid are used for amplifying the primary amplified product 602. The primary amplified product 602 is obtained from an earlier amplification using the primer 606 and a primer which targets a region further upstream of the specific nucleic acid sequence targeted by the probe 604. The FAM-labelled probe 604 and the biotin-labelled primer 606 provide a point of initiation for generation of double stranded nucleic acid via extension from the 3' ends of the probe and primer by DNA polymerases (as indicated by reference arrows 608 and 610). As the amplification reaction progresses, a secondary amplified product/ secondary amplicon 612 containing both FAM and biotin labels is formed. The secondary amplicon 612 can be detected using assays such as lateral flow immunoassays.

Fig. 7 is a schematic diagram showing a lateral flow strip 700 in an exemplary embodiment. The lateral flow strip 700 comprises a capillary bed 702, a sample pad 704 for loading a test sample fluid disposed adjacent to the capillary bed 702, a test line 706 and a control line 708 disposed on the capillary bed 702. The sample pad 704 is incorporated with nano-colloidal gold particles 710 with an antibody which identifies one of the labels on an amplified product e.g. anti-FAM or anti-biotin antibodies 712 immobilised on the surface of the nano-colloidal

gold particles 710. It will be appreciated that any other suitable particles e.g. carbon nanoparticles may also be used in place of gold nanoparticles. The test line 706 comprises capture molecules 714 in the form of anti-biotin/streptavidin or anti-FAM (depending on whether the colloidal nanoparticles are conjugated with anti-FAM or anti-biotin respectively) immobilised on the capillary bed 702 and is configured to bind to biotin molecules. The control line 708 comprises antibodies in the form of anti-rabbit IgG (immunoglobulin G) 716 immobilised on the capillary bed 702, which captures any particles and thereby shows that the lateral flow strip assay has run correctly. In the exemplary embodiment, a second test line (not shown) may be provided. The second test line may comprise a suitable antibody, such as anti-digoxigenin for simultaneously capturing a second target of interest using the corresponding primers/probe with distinct tags (in this case, digoxigenin, DIG) to produced double-labelled amplicons with DIG-biotin tags, as opposed to FAM-biotin tags amplicon (first target of interest).

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In use, a test sample fluid containing a double labelled amplicon e.g. FAM/biotin labelled amplicon 718 is loaded onto the sample pad 704. The double labelled amplicon 718 interacts with the nano-colloidal gold particles 714 and forms a complex 720 via binding of the anti-FAM antibodies 712 to the FAM label on the double labelled amplicon 718. Once soaked, the test sample fluid migrates along the capillary bed 702 towards the test line 706 and control line 708.

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Fig. 8 is a schematic diagram showing a complex 800 formed between an amplified DNA product 802 and a nanocolloidal gold particle 804 in an exemplary embodiment. The amplified DNA product 802 is doubly labelled with FAM 806 on a first DNA strand 808 and biotin 810 on a second opposing DNA strand 812. The complex 800 is formed by adding a sample suspected of containing the amplified DNA product 802 to a pad (not shown) incorporated/ soaked in the nanocolloidal gold particle 804. Anti-FAM antibodies 814 are immobilised on the nano-colloidal gold particle 804 and these anti-FAM antibodies 814 bind specifically to the FAM 806 label (antigen) on the amplified DNA product 802. In

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other embodiments, other antibodies such as anti-biotin or anti-DIG antibodies may be immobilised on the nano-colloidal particles.

Fig. 9 is a schematic diagram showing a test lane 902 segment of a lateral flow strip 900 in an exemplary embodiment. The test lane 902 of the lateral flow strip 900 comprises streptavidin molecules 904 immobilised on a capillary bed 906. The streptavidin molecules 904 have a high affinity for binding to biotin. With a dissociation constant (K_d) on the order of $\approx 10^{-14}$ mol/L, the binding of biotin to streptavidin is one of the strongest non-covalent interactions known in nature. Accordingly, an amplified DNA product 908 labelled with biotin 910 at one end of the DNA strand will be captured by the streptavidin molecules 904 as the amplified DNA product 908 flows along the capillary bed 906. The amplified DNA product 908 is complexed with a nano-colloidal gold particle 912 via anti-FAM antibody 914 binding to a FAM molecule 916 on an opposing DNA strand. As more double labelled amplified DNA products 908 reach the test lane 902 and are captured by the streptavidin molecules 904, aggregation of gold or carbon nanoparticles will occur, resulting in a visible line appearing on the test lane 902, thus allowing the test to be read as a positive. For the gold nanoparticles (but not for the carbon particles), a colour change will occur from red to blue when the gold nanoparticles are aggregated. In other embodiments, other antibodies may be immobilised on the capillary bed depending on the type of amplified DNA products to be detected. For example, anti-FAM and/or anti-DIG antibodies may be immobilised on the nano-colloidal particles for detection of one or two different amplified DNA products.

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Fig. 10 is a schematic diagram showing the control lane 1002 segment of a lateral flow strip 1000 in an exemplary embodiment. The control lane 1002 of the lateral flow strip 1000 comprises antibodies e.g. anti-rabbit IgG (immunoglobulin G) antibodies 1004 immobilised on a capillary bed 1006. Anti-IgG 1004 acts as a non-specific control and turns positive regardless of the presence or absence of target analyte e.g. amplified DNA products within the test sample. As shown in Fig. 10, the anti-IgG 1004 binds to a nano-colloidal gold

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particle 1008 via anti-FAM antibodies 1010 disposed on the surface of the nano-colloidal gold particle 1008. As more nano-colloidal gold particles 1008 reach the control lane 1002 and binds to the anti-IgG 1004, aggregation of gold or carbon nanoparticles will occur, resulting in a visible line appearing on the control lane 1002. In other embodiments, other antibodies such as anti-biotin or anti-DIG antibodies may be disposed on the surface of the nano-colloidal gold particle.

EXAMPLES

Example embodiments of the disclosure will be better understood and readily apparent to one of ordinary skill in the art from the following examples, tables and if applicable, in conjunction with the figures.

Example 1 - Materials and reagents

Table 2 below provides a list of the materials and reagents for performing on-site detection of pathogens using the on-site detection system.

Table 2. Materials and reagents for performing on-site detection of pathogens

Materials	Quantity
Portable (battery-operated) heating system	1
100 mL Disposable Syringe (zsq07) (http://www.jxhd.cn/products_detail/productId=123.html ; ITS Science & Medical Pte Ltd)	1
CellTrap Filter (CT22) (http://www.mem-teq.co.uk/index.php/technical-info/ ; AuRUM MEDICARE PTE LTD)	1
1mL Disposable Syringe (BD 309628) (Zuellig Pharma Pte Ltd Singapore)	3
Luer-Lock Tip Cap (BD 308341) (Zuellig Pharma Pte Ltd Singapore)	3
Plastic tips /nozzles for syringe (AD3120PP-B)	6

Adhesive Dispensing Ltd (UK)	
1.5mL microfuge Tube (1210-00S) (SSI, USA ; Practical Mediscience Pte Ltd)	3
5mL Eppendorf Tube (EPPE0030119.401) (Eppendorf; VWR Singapore Pte Ltd)	3
Lateral Flow Kit (PCRD3KIT Abingdon Health, UK; TwistDx Ltd. UK)	3
Reagents	Volume
Premix solution (refer to Table 4)	150 μ L (x3)
Buffer solution (PCRD3KIT Abingdon Health, UK; TwistDx Ltd. UK)	2800 μ L (x3)

Table 3 below provides exemplary concentrations and volumes of reagents used in recombinase polymerase amplification.

5 Table 3. Concentrations and volumes of reagents in 150 μ L of premix solution

Reagents	Volume (μ L)
Primer A (10 μ M) (freeze-dried)	8.4 μ L
Primer B (10 μ M) (freeze-dried)	8.4 μ L
Probe (10 μ M) (freeze-dried)	2.4 μ L
Positive control DNA (freeze-dried)	8 μ L
Freeze-dried enzyme mixture	4 x reaction equivalent
Rehydration buffer	118 μ L
MgOAc (280 mM)	10 μ L
Water (PCR-grade)	Variable volume

The positive control DNA are *Pseudomonas aeruginosa* (ATCC 27853), *Legionella pneumophila* (ATCC 33152), and *Enterococcus faecium* (ATCC 35667) purified using DNeasy Blood & Tissue Kit (Qiagen).

Example 2 - Measuring performance metrics of amplification module

The performance of the amplification module (isothermal recombinase polymerase amplification) of on-site detection system was evaluated against different kinds of field samples. A total of 60 tests were performed on water samples from cooling towers, spa and water playground (30 tests using a Ps formulation for detecting *Pseudomonas aeruginosa* and another 30 tests using a Lg formulation for detecting *Legionella pneumophila*). The test results were compared against qPCR (quantitative polymerase chain reaction) performed on the same water samples.

Performance parameters such as sensitivity, specificity, negative predictive value, positive predictive value, false negative rate and false positive rate are calculated based on the following formulae.

A = true positive

B = false positive

C = false negative

D = true negative

Test sensitivity = $A / (A + C)$

Test specificity = $D / (B + D)$

Positive predictive value (PPV) = $A / (A + B)$

Negative predictive value (NPV) = $D / (C + D)$

False negative rate = $C / \text{total number of samples}$

False positive rate = $B / \text{total number of samples}$

Table 4 below summarises the performance of isothermal amplification against qPCR.

Table 4. Performance metrics of isothermal amplification against qPCR

Sensitivity	82.4%
Specificity	>75.1%
Negative predictive value (NPV)	>88%
Positive predictive value (NPV)	>79%
False negative rate	<10%

False positive rate	<13%
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Example 3 - Comparison of recombinase polymerase amplification with quantitative polymerase chain reaction

5 Fig. 11 is a photograph of lateral flow strip test results for detection of cells treated/untreated with propidium monoazide (PMA) in an exemplary embodiment. Samples of cells containing a target bacteria were treated with PMA to bind to DNA of dead cells, prior to amplification using isothermal amplification.

10 Sample 1100 is a negative control (NTC, no template control) with no amplified products belonging to the target bacteria. A visible line is shown on the control lane 1110 showing that the test has run correctly.

15 Samples 1102 and 1104 contain a mixture of live and dead cells of the target bacteria. Sample 1102 was not treated with PMA prior to isothermal amplification and therefore the amplified products of the target bacteria may belong to both live and dead cells. For sample 1102, a visible line is shown on the control lane 1110 and test line 1112, indicating the presence of the target bacteria in the sample. Sample 1104 was treated with PMA prior to isothermal
20 amplification and therefore the amplified products of the target bacteria belongs to live cells only. For sample 1104, a visible line is shown on the control lane 1110 and test line 1112, indicating the presence of the target bacteria in the sample.

25 Samples 1106 and 1108 contain dead cells belonging the the target bacteria. Sample 1106 was not treated with PMA prior to isothermal amplification and therefore the DNA of dead cells of the target bacteria was amplified. For sample 1106, a visible line is shown on the control lane 1110 and test line 1112, indicating the presence of dead target bacteria in the sample. Sample 1108 was treated with PMA prior to isothermal amplification and therefore the DNA of dead
30 cells of the target bacteria was rendered incapable of amplification. For sample

1108, a visible line is shown on the control lane 1110, indicating the presence of dead target bacteria in the sample.

As a comparison, qPCR was performed on groups of samples containing
 5 (I) live cells of the target bacteria without PMA treatment; (II) live cells of the target bacteria with PMA treatment; (III) dead cells of the target bacteria; and (IV) dead cells of the target bacteria with PMA treatment.

Fig. 12 is a graph showing relative fluorescence unit (RFU) against number
 10 of amplification cycles of target DNA using qPCR in an exemplary embodiment. In qPCR, the accumulation of amplicons is detected and quantified periodically by fluorescence at every cycle. In the initial cycles of PCR, the fluorescent signal emitting from the sample is low and is considered to be background signal, defining a baseline 1200 for the amplification of the sample. As more amplicons
 15 accumulate in the later cycles, the signal gradually rises above the baseline 1200. As shown in Fig. 12, a threshold line 1202 is set above the baseline 1200, indicating an equal amount of amplicons in different samples. In general, the intensity of the fluorescence signal is directly correlated to the concentration of amplicons during the linear phase of the reaction, and becomes non-linear during
 20 the later stage of the reaction (i.e. change in RFU is not directly proportional to the amount of amplicons). The threshold line 1202 by default indicates the RFU signal at mean background noise plus three (3) standard deviations, and represents the initial rate of amplification (within linearity) of the reaction. Each reaction (depending on the amount of target/template DNA originally present),
 25 will reach the threshold point at different times (in this case, at different cycle numbers). A CT (also known as Cq) is defined as the number of cycles required for the fluorescence signal to cross the threshold. A larger amount of target DNA present in the sample would lead to faster amplification, thus resulting in the RFU reaching threshold point earlier. The opposite is true. The rate at which the RFU
 30 reaches threshold point is directly proportional to the amount of template/target present. Meaning to say, the Cq number and the original amount of template has a linear relationship, i.e. negatively correlated.

Table 5 below summarises the quantitative cycle (Cq) values of the different groups of sample. Cq number is the cycle number at which fluorescence from target amplification exceeds the threshold 1202.

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Table 5. Quantitative cycle (Cq) values of the sample groups during qPCR

Group	Cq
I) Live cells	34.72
II) Live cells + PMA	35.29
III) Dead cells	35.38
IV) Dead cells + PMA	N/A
NTC (no template control)	N/A

As shown in Fig. 12, the cluster of lines pointed by reference numeral 1204 belong to the groups (I) live cells of the target bacteria without PMA treatment; (II) live cells of the target bacteria with PMA treatment; and (III) dead cells of the target bacteria. This indicates that the DNAs of target bacteria in these groups have been amplified. Group (IV) samples (i.e. dead cells of the target bacteria with PMA treatment) and the negative control sample were not amplified above the threshold 1202 during qPCR, as indicated by reference numerals 1206 and 1208 respectively.

Fig. 13 is a graph showing melt curve analysis of the qPCR products in an exemplary embodiment. At the end of qPCR, a melt curve analysis of the products was performed. The melt curve measures the changes of fluorescence intensity of a sample when heated. When the temperature is increased, the fluorescent signal decreases gradually and becomes more abrupt at certain temperatures. The decrease in fluorescent signal is due to temperature-dependent denaturing of duplex DNA which leads to dissociation of the fluorescent dye (e.g. SYBR-green dye). As shown in Fig. 13, the cluster of lines pointed by reference numeral 1300 belong to the groups (I) live cells of the target bacteria without PMA treatment; (II) live cells of the target bacteria with PMA

treatment; and (III) dead cells of the target bacteria. Group (IV) samples (i.e. dead cells of the target bacteria with PMA treatment) and the negative control sample (as indicated by reference numerals 1302 and 1304 respectively) were not amplified above the threshold level and therefore there is negligible qPCR product for melt curve analysis. Part of the RFU signal for the negative control sample (as indicated by reference numeral 1304) is overlapped within the RFU signals for Group (IV) samples (as indicated by reference numeral 1302) in the region from 76°C to 95°C.

Fig. 14 is a graph showing melt peak analysis of the qPCR products in an exemplary embodiment. To compare and visualise the multiple melt curves of Fig. 13, the first negative derivative ($-d(RFU)/dT$) of the melt curve is plotted to identify distinct peaks. As shown in Fig. 14, the cluster of lines pointed by reference numeral 1402 belong to the groups (I) live cells of the target bacteria without PMA treatment; (II) live cells of the target bacteria with PMA treatment; and (III) dead cells of the target bacteria. Distinct peaks 1408 are observed from the melt peak curves of Groups (I)-(III) (cluster of lines as indicated by reference numeral 1402), indicating the formation of at least one PCR product. Group (IV) samples (i.e. dead cells of the target bacteria with PMA treatment) and the negative control sample (as indicated by reference numerals 1404 and 1406 respectively) were not amplified above the threshold level and therefore there is negligible qPCR product for melt peak analysis. Part of the signal for the negative control sample (as indicated by reference numeral 1406) is overlapped within the signals for Group (IV) samples (as indicated by reference numeral 1404) in the region from 79°C to 95°C.

The above qPCR results are in agreement with the isothermal amplification and lateral flow strip test results of Fig. 11. This shows that the on-site system may be capable of achieving the same accurate results as a laboratory test using sophisticated PCR equipment.

The results of the lateral flow strip test demonstrate the effectiveness of PMA treatment in binding to DNA of non-viable cells (i.e. dead cells or cells with compromised cell membranes).

5 Example 4 - Recombinase polymerase amplification - most probable
number experiment (RPA-MPN)

Fig. 15 is a flowchart 1500 showing a method of performing a RPA-MPN experiment in an exemplary embodiment. The most probable number method is
10 a method of getting quantitative data on concentrations of discrete items from positive/negative (incidence) data. At step 1502, *Pseudomonas aeruginosa* bacterium was cultured overnight under suitable cell culture conditions. At step 1504, a sample of the cultured bacterium was harvested and serial dilution was performed with various orders of magnitude (10^{-1} , 10^{-2} ... 10^{-7}). At step 1506, the
15 diluted samples of bacterium were applied on a spread plate. The spread plate method is a technique to plate a liquid sample containing bacteria so that the bacteria are easy to count and isolate. A successful spread plate will have a countable number of isolated bacterial colonies evenly distributed on the plate. The applied spread plates were left overnight. At step 1508, the number of colony
20 forming units (CFU) on the spread plates was determined.

At step 1510, suitable diluted bacteria suspension was picked and spiked in 100 ml of water. At step 1512, 100 ml of water spiked with bacteria was filtered and concentrated using CellTrap (dead-end cross-flow hybrid filter) to obtain a
25 sample of bacteria. At step 1514, the sample of bacteria was lysed for 5 minutes at 95°C and another serial dilution was performed to dilute the sample into the following orders of magnitude (10^{-1} , 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} and 10^{-6}). At step 1516, RPA and lateral flow strip detection were performed on the diluted samples. It should be noted that the RPA experiment was conducted on the same day as the
30 spread plate of step 1506 to ensure that the CFU count arrived the following day is the actual CFU count used during the experiment.

At step 1518, the results of the lateral flow strip detection were obtained. At step 1520, the results of the lateral flow strip detection were verified against the spread plate count in step 1508. The results show that a dilution of 10^{-1} (i.e. 10 times dilution) contains 100 or more CFUs). Therefore, the original sample contains 1000 or more CFUs.

Fig. 16 is a photograph of lateral flow strip test results for the RPA-MPN experiment in an exemplary embodiment. The results of samples 1600 to 1612 are summarised in Table 6 below.

Table 6. Results and interpretation of lateral flow strip test

Sample reference numeral	1600	1602	1604	1606	1608	1610	1612
Dilution factor	NTC	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Interpretation	N/A	≥ 10 CFU	≥ 1 CFU	0 CFU	0 CFU	0 CFU	0 CFU

Fig. 17 is a photograph of spread plates for the RPA-MPN experiment in an exemplary embodiment. The results of samples 1700 to 1708 and 1710 to 1718 are summarised in Table 7 below.

Table 7. Results and interpretation of spread plate method

Dilution factor	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Sample reference numeral	1700	1702	1704	1706	1708
CFU count	TNTC	TNTC	215	21	2
Sample reference numeral	1710	1712	1714	1716	1718
CFU count	TNTC	TNTC	235	24	2
Average CFU count	TNTC	TNTC	225	22.5	2

TNTC: too numerous to count

Based on the above results in Table 6, a sample which is diluted by 10 times contains 10 or more CFUs. Therefore, the original sample contains 100 or more CFUs. Based on the above results in Table 7, the average CFU count is 2.25 x 10⁵ CFU/μL and the actual amount of bacteria spiked into the water is 225 CFU. Taking into account the loss of cells due to CellTrap filtration, the overall MPN is within the same order of magnitude.

Example 5 – Isothermal DNA amplification (fluorescence detection)

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Fig. 18 is a graph showing relative fluorescence unit (RFU) against time in a real-time DNA amplification in an exemplary embodiment. Isothermal DNA amplification was conducted in the presence of specific primers and fluorescence probe for *Enterococcus faecium*. The probe used in the amplification is based on SEQ ID NO. 15:

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5'-ACTCTGTTGTTAGAGAAGAACAAGGATGAGAG/iFluorT/A/idSp/C/iBHQ-1dT/GTTCATCCTTGACG/3Phos/-3'.

In Fig. 18, the amplification curve of the target DNA sequence of *Enterococcus faecium* is represented by reference numeral 1800, the amplification curve of the NTC (no template control) is represented by reference numeral 1802, and the amplification curve of the target DNA sequence of *E. coli* is represented by reference numeral 1804. DNA amplification of the target DNA sequence of *Enterococcus faecium* resulted in an associated increase in fluorescence detected. The NTC group had no change in fluorescence over time. *E. coli* was not the target bacteria of interest and as a result, the DNA sequence of *E. coli* was not amplified and the amplification curve 1804 saw negligible change in fluorescence.

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Fig. 19 is a graph showing relative fluorescence unit (RFU) against time in a real-time DNA amplification of samples with different initial concentrations of a DNA template in an exemplary embodiment. The template used was the DNA

sequence of *Enterococcus faecium*. As shown in Fig. 19, the higher the initial concentration of the DNA template (measured in CFU, colony forming unit), the shorter the time taken for the fluorescence to cross the signal threshold (indicated by the reference numeral 1900).

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Fig. 20 is a graph showing Log CFU (*E. faecium*) against fluorescence signal (threshold point) in an exemplary embodiment. Threshold point corresponds to the initial increase in fluorescence signal (which is 3 S.D. + background fluorescence signal, where S.D. is standard deviation). As shown in Fig. 20, the higher the initial concentration of the DNA template, the faster it takes to reach threshold point. The change in Log CFU and threshold point appears to follow a negative linear correlation ($R^2 = 0.9388$, close to 1).

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Fig. 21 is a partial nucleic acid sequence of *Pseudomonas aeruginosa* algD gene for GDP-mannose dehydrogenase at positions 121 to 660 in an exemplary embodiment. It is appreciated that a double-stranded DNA consists of a sense and an antisense strand. In Fig. 21, only the sense strand of the partial nucleic acid sequence of *Pseudomonas aeruginosa* algD gene for GDP-mannose dehydrogenase is shown. Fig. 21 also shows exemplary nucleic acid sequences of primers and probes that may be used to amplify a target region on the nucleic acid sequence. The primers and probes are based on SEQ ID NO. 1 to 5, with attached molecules/labels indicated at the 5'/3' ends and/or superscript at various positions of the nucleotide sequences as shown in the figure. In Fig. 21, SEQ ID NO.1, which is the forward primer, together with SEQ ID NO. 4 and SEQ ID NO. 5 (both of which are the probes) are shown as being directly aligned to the sense strand in a base-to-base manner. During amplification, the forward primer, including the probe, will each bind to their respective complementary sequence on the antisense strand which is not shown. On the other hand, the reverse primers (SEQ ID NO. 2 and SEQ ID NO. 3) are shown as being directly aligned to their complementary sequences on the sense strand.

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To amplify a target region of the nucleic acid sequence of the bacteria, a RPA assay using a two-step amplification process may be used. In the first step, SEQ ID NO. 1 may be used as a forward primer together with either SEQ ID NO. 2 or SEQ ID NO. 3 acting as reverse primers, to form a primary amplification product. In the second step, SEQ ID NO. 4 or SEQ ID NO. 5 may be used as probes (acting as forward primers) together with either SEQ ID NO. 2 or SEQ ID NO. 3 acting as reverse primers, to form a secondary amplification product. In the exemplary embodiment, the primary amplification product has 531 bp (base pairs) and the secondary amplification product has 181 base pairs. In the exemplary embodiment, SEQ ID NO. 4 may be used as a lateral flow probe for amplifying target nucleic acid sequences for subsequent detection using a lateral flow system. SEQ ID NO. 5 may be used as a fluorescence labelled probe for amplifying target nucleic acid sequences for subsequent detection using a fluorescence detection system.

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Fig. 22 is a partial nucleic acid sequence of *Legionella pneumophila* 16S ribosomal RNA at positions 421 to 840 in an exemplary embodiment. It is appreciated that a double-stranded DNA consists of a sense and an antisense strand. In Fig. 22, only the sense strand of the partial nucleic acid sequence of *Legionella pneumophila* 16S ribosomal RNA is shown. Fig. 22 also shows exemplary nucleic acid sequences of primers and probes that may be used to amplify a target region on the nucleic acid sequence. The primers and probes are based on SEQ ID NO. 6 to 10, with attached molecules/labels indicated at the 5'/3' ends and/or superscript at various positions of the nucleotide sequences as shown in the figure. In Fig. 22, SEQ ID NO. 6, which is the forward primer, together with SEQ ID NO. 9 and SEQ ID NO. 10 (both of which are the probes) are shown as being directly aligned to the sense strand in a base-to-base manner. During amplification, the forward primer, including the probe, will each bind to their respective complementary sequence on the antisense strand which is not shown. On the other hand, the reverse primers (SEQ ID NO. 7 and SEQ ID NO. 8) are shown as being directly aligned to their complementary sequences on the sense strand.

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To amplify a target region of the nucleic acid sequence of the bacteria, a RPA assay using a two-step amplification process may be used. In the first step, SEQ ID NO. 6 may be used as a forward primer together with either SEQ ID NO. 7 or SEQ ID NO. 8 acting as reverse primers, to form a primary amplification product. In the second step, SEQ ID NO. 9 or SEQ ID NO. 10 may be used as probes (acting as forward primers) together with either SEQ ID NO. 7 or SEQ ID NO. 8 acting as reverse primers, to form a secondary amplification product. In the exemplary embodiment, the primary amplification product has 392 bp and the secondary amplification product has 182 base pairs. In the exemplary embodiment, SEQ ID NO. 9 may be used as a lateral flow probe for amplifying target nucleic acid sequences for subsequent detection using a lateral flow system. SEQ ID NO. 10 may be used as a fluorescence labelled probe for amplifying target nucleic acid sequences for subsequent detection using a fluorescence detection system.

Fig. 23 is a partial nucleic acid sequence of *Enterococcus faecium* strain (ATCC 19434) 16S ribosomal RNA gene at positions 181 to 600 in an exemplary embodiment. It is appreciated that a double-stranded DNA consists of a sense and an antisense strand. In Fig. 23, only the sense strand of the partial nucleic acid sequence of *Enterococcus faecium* strain (ATCC 19434) 16S ribosomal RNA gene is shown. Fig. 23 also shows exemplary nucleic acid sequences of primers and probes that may be used to amplify a target region on the nucleic acid sequence. The primers and probes are based on SEQ ID NO. 11 to 15, with attached molecules/labels indicated at the 5'/3' ends and/or superscript at various positions of the nucleotide sequences as shown in the figure. In Fig. 23, SEQ ID NO. 11, which is the forward primer, together with SEQ ID NO. 14 and SEQ ID NO. 15 (both of which are the probes) are shown as being directly aligned to the sense strand in a base-to-base manner. During amplification, the forward primer, including the probe, will each bind to their respective complementary sequence on the antisense strand which is not shown. On the other hand, the reverse

primers (SEQ ID NO. 12 and SEQ ID NO. 13) are shown as being directly aligned to their complementary sequences on the sense strand.

To amplify a target region of the nucleic acid sequence of the bacteria, a RPA assay using a two-step amplification process may be used. In the first step, SEQ ID NO. 11 may be used as a forward primer together with either SEQ ID NO. 12 or SEQ ID NO. 13 acting as reverse primers, to form a primary amplification product. In the second step, SEQ ID NO. 14 or SEQ ID NO. 15 may be used as probes (acting as forward primers) together with either SEQ ID NO. 12 or SEQ ID NO. 13 acting as reverse primers, to form a secondary amplification product. In the exemplary embodiment, the primary amplification product has 371 bp and the secondary amplification product has 181 base pairs. In the exemplary embodiment, SEQ ID NO. 14 may be used as a lateral flow probe for amplifying target nucleic acid sequences for subsequent detection using a lateral flow system. SEQ ID NO. 15 may be used as a fluorescence labelled probe for amplifying target nucleic acid sequences for subsequent detection using a fluorescence detection system.

20 APPLICATIONS

Embodiments of the disclosure provided herein may provide an on-site system and method for detecting pathogens in a liquid sample. In various embodiments, the on-site system comprises a filtration module to obtain a sample population of pathogens from the liquid sample, an amplification module to amplify one or more target biological elements present in one or more target species of pathogens in the sample population of pathogens; and a detection module configured to detect presence of amplified products of the one or more target biological elements present in the one or more target species of pathogens.

Various embodiments of the present disclosure provide a lab-in-a-suitcase capable of performing on-site detection of water pathogens under 1 hour by incorporating the mechanisms of concentration and detection. For concentration, a hybrid dead-end cross-flow filtration system may be used to concentrate a target bacteria. For detection, recombinase polymerase amplification may be used to confirm the target bacteria through its signature DNA. Various embodiments of the present disclosure provide unique formulations of reagents, e.g. Ps Formulation and Lg Formulation, to detect *Pseudomonas aeruginosa* and *Legionella pneumophila* respectively. Various embodiments of the present disclosure also provide another unique formulation of reagents, Ps-Lg Formulation formed by mixing of Ps and Lg formulations, which can be able to detect both *Pseudomonas aeruginosa* and *Legionella pneumophila* concurrently in a multiplexing reaction. In various embodiments, the formulations (Ps, Lg, Ps-Lg B) are specially formulated and freeze-dried to allow for ambient temperature storage and are ready to use upon a single mixing step.

Various embodiments of the on-site system and method may optionally comprise a treatment module to render one or more biological elements in non-viable pathogens incapable of amplification. For example, the treatment module may comprise propidium monoazide (PMA) treatment. Various embodiments of the present disclosure provide a PMA formulation where detection will only target viable bacterium DNA, and not free floating DNA or DNA in non-viable bacteria. In various embodiments, the PMA formulation is specially formulated and freeze-dried to allow for ambient temperature storage and is ready to use upon a single mixing step.

Various embodiments of the present disclosure also provide a method, formulation and test device/apparatus for rapid on-site detection of specific waterborne bacteria of interest (primary function) with the sensitivity of a drinking water standards and analysis and differentiation of live and dead bacteria (secondary function). In various embodiments, the test reagents

required are specially formulated and prepared such that they can be stably stored for long period of time at ambient temperature, without the need for refrigeration or any special storage condition. Moreover, in various embodiments, the test reagents are ready to use upon a single mixing step.

- 5 Various embodiments of the present disclosure are not intended to replace conventional testing (culture methods) and instead, will be used to complement by providing a quick qualitative screening (YES/NO).

10 Various embodiments of the present disclosure provide a cheaper and cost effective option of detecting the presence of pathogens. Advantageously, the cost of using the on-site detection system is relatively low and competitive as compared to culture methods.

15 Various embodiments of the present disclosure provide an on-site detection system which is portable and suitable for on-site/ out-field testing. This advantageously provides an on-site system and method of point-of-care water sampling and testing. The on-site detection system is also relatively easy to use and is suitable for non-technical personnel to carry out water sampling and testing. Advantageously, the use of micropipettors, which is a commonly required
20 instrument for most biological experiments or detection processes, may be omitted, thereby significantly reducing cost. Accordingly, in some embodiments, the methods disclosed herein do not require the use of micropipettors. Various embodiments of the present disclosure also contrast with laboratory-based molecular detection methods such as PCR (Polymerase Chain Reaction) which
25 involve higher associated cost and higher level of technical expertise required to perform the detection method.

Even more advantageously, the on-site detection system may be capable of detecting the presence of pathogens in a relatively short time of about 45 to 90
30 minutes, thereby providing rapid test-to-result as compared to the cell culture method which typically takes more than 2 days. In addition, the on-site detection

system may have excellent sensitivity with a detection limit of about 0.8-1.5 CFU/100 ml which is comparable to the cell culture method.

5 The above characteristics enable the on-site detection system to be used
in point-of-care-testing, on-site testing, rapid detection, microbial water
testing, water quality monitoring, water analysis, water management, water
sampling, and environmental surveillance and the like. It can be used for
routine testing and serve as an early-warning system to complement existing
culture methods. It can be routinely deployed as an active and early warning
10 system which current surveillance system lacks.

It will be appreciated by a person skilled in the art that other variations
and/or modifications may be made to the embodiments disclosed herein
without departing from the spirit or scope of the disclosure as broadly
15 described. The present embodiments are, therefore, to be considered in all
respects to be illustrative and not restrictive.

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CLAIMS

1. A portable on-site detection system for detecting pathogens in a small volume of a liquid sample of no more than 100 ml on-site, the system
5 comprising:

a filtration module comprising a mixture of dead-end filtration elements and cross-flow filtration elements configured to obtain a sample population of pathogens from the liquid sample,

an amplification module configured to amplify one or more target
10 biological elements present in one or more target species of pathogens in the sample population of pathogens; and

a detection module configured to detect presence of amplified products of the one or more target biological elements present in the one or more target species of pathogens,

15 wherein a positive reading on the detection module indicates the presence of the one or more target species of pathogens, and

wherein the system is configured to perform detection of pathogens on-site in less than 90 minutes.

20 2. The system of claim 1, further comprising,

a treatment module comprising a composition for preferentially interacting with one or more biological elements present in non-viable pathogens in the sample population of pathogens to render said one or more biological elements incapable of amplification.

25

3. The system of claim 1 or 2, wherein the pathogens are selected from the group consisting of bacteria, viruses, parasites and fungi.

4. The system of any one of claims 1 to 2, wherein the one or more biological elements comprise DNA.

5 5. The system of any one of claims 2 to 4, wherein the composition for preferentially interacting with one or more biological elements comprises propidium monoazide.

6. The system of any one of claims 1 to 5, wherein the amplification
10 module comprises recombinase polymerase amplification of the one or more target DNAs from the one or more target species of pathogens in the sample population of pathogens.

7. The system of any one of claims 1 to 6, wherein the amplification
15 module comprises a probe and at least one primer, wherein the probe and the at least one primer comprise a nucleotide sequence having at least 80% sequence identity to a sequence selected from the group consisting of SEQ ID NO. 1 to SEQ ID NO. 5 for detecting *Pseudomonas aeruginosa*.

20 8. The system of any one of claims 1 to 7, wherein the amplification module comprises a probe and at least one primer, wherein the probe and the at least one primer comprise a nucleotide sequence having at least 80% sequence identity to a sequence selected from the group consisting of SEQ ID NO. 6 to SEQ ID NO. 10 for detecting *Legionella pneumophila*.

25

9. The system of any one of claims 1 to 8, wherein the amplification module comprises a probe and at least one primer, wherein the probe and

the at least one primer comprise a nucleotide sequence having at least 80% sequence identity to a sequence selected from the group consisting of SEQ ID NO. 11 to SEQ ID NO. 15 for detecting *Enterococcus* species.

5 10. The system of any one of claims 1 to 9, wherein the amplification module further comprises reagents comprising a freeze-dried enzyme mixture, a rehydration buffer, a solution of magnesium acetate, a labelled probe, a positive control template DNA and water, wherein the reagents are formulated for storage at ambient temperature and ready to use upon a single
10 mixing step.

 11. The system of any one of claims 1 to 10, wherein the amplification module further comprises one or more portable, battery-operated heating devices for providing one or more operating temperatures
15 to perform amplification.

 12. The system of any one of claims 1 to 11, wherein the detection module comprises a lateral flow strip configured to detect labelled amplified products.

20

 13. The system of any one of claims 1 to 11, wherein the detection module comprises a fluorescence detector configured to detect fluorescence-labelled amplified products.

25 14. The system of any one of claims 1 to 13, wherein the system has a sensitivity of at least 80%.

15. The system of any one of claims 1 to 14, wherein the system has a specificity of at least 75%.

16. The system of any one of claims 1 to 15, wherein said system is
5 capable of detecting pathogens in the liquid sample at a concentration range from 0.8 to 1.5 CFU per 100 ml.

17. A method for detecting pathogens in a small volume of a liquid sample of no more than 100 ml on-site in less than 90 minutes, the method
10 comprising,

filtering the liquid sample with the filtration module of the detection system of any one of claims 1 to 16;

amplifying one or more target biological elements present in one or more target species of pathogens in the sample population of pathogens with
15 the amplification module of the detection system of any one of claims 1 to 16;
and

detecting the presence of amplified products of the one or more target biological elements present in the one or more target species of pathogens with the detection module of the detection system of any one of claims 1 to
20 16.

18. The method of claim 17, further comprising treating the sample population of pathogens with a composition for preferentially interacting with one or more biological elements present in non-viable pathogens in the
25 sample population of pathogens to render said one or more biological elements incapable of amplification.

19. The method of claim 17 or 18, wherein the pathogens are selected from the group consisting of bacteria, virus, parasites and fungi.

20. The method of any one of claims 17 to 19, wherein the one or
5 more biological elements comprise DNA.

21. The method of any one of claims 18 to 20, wherein the step of treating the sample population of pathogens with a composition comprises exposing the sample population of pathogens to propidium monoazide.
10

22. The method of any one of claims 17 to 21, wherein the step of amplifying comprises performing recombinase polymerase amplification of the one or more target biological elements from the one or more target species of pathogens in the sample population of pathogens.

23. The method of any one of claims 17 to 22, wherein the step of amplifying comprises using a probe and at least one primer, wherein the probe and the at least one primer comprise a nucleotide sequence having at least 80% sequence identity to a sequence selected from the group
15 consisting of SEQ ID NO. 1 to SEQ ID NO. 5 for detecting *Pseudomonas aeruginosa*.
20

24. The method of any one of claims 17 to 23, wherein the step of amplifying comprises using a probe and at least one primer, wherein the
25 probe and the at least one primer comprise a nucleotide sequence having at least 80% sequence identity to a sequence selected from the group

consisting of SEQ ID NO. 6 to SEQ ID NO. 10 for detecting *Legionella pneumophila*.

25. The method of any one of claims 17 to 24, wherein the step of
5 amplifying comprises using a probe and at least one primer, wherein the probe and the at least one primer comprise a nucleotide sequence having at least 80% sequence identity to a sequence selected from the group consisting of SEQ ID NO. 11 to SEQ ID NO. 15 for detecting *Enterococcus* species.

10

26. The method of any one of claims 17 to 25, wherein the step of amplifying comprises using reagents comprising a freeze-dried enzyme mixture, a rehydration buffer, a solution of magnesium acetate, a labelled probe, a positive control template DNA and water, wherein the reagents are
15 formulated for storage at ambient temperature and ready to use upon a single mixing step.

27. The method of any one of claims 17 to 26, wherein the step of amplifying comprises providing one or more operating temperatures with one
20 or more portable, battery-operated heating devices.

28. The method of any one of claims 17 to 27, wherein the step of detecting comprises using a lateral flow strip configured to detect labelled amplified products.

25

29. The method of any one of claims 17 to 27, wherein the step of detecting comprises using a fluorescence detector configured to detect fluorescence-labelled amplified products.

5 30. The method of any one of claims 17 to 29, wherein the method has a sensitivity of at least 80%.

31. The method of any one of claims 17 to 30, wherein the method has a specificity of at least 75%.

10

32. The method of any one of claims 17 to 31, wherein said method is capable of detecting pathogens in the liquid sample at a concentration range from 0.8 to 1.5 CFU per 100 ml.

15 33. The system of any one of claims 7 to 9 or a method of any one of claims 23 to 25, wherein said at least one pair of primers comprises a pair of primers.